ANGIOGENESIS RELATED MOLECULES

<u>Technical field</u>

5   The present invention relates to the field of angiogenesis, and more specifically to novel molecules, such as proteins and peptides, whereby novel anti-angiogenic substances may be developed. The invention also relates to methods for developing such substances.

<u>Background</u>

Almost all of the tissue of a mammalian body comprises a fine mesh of very thin
10   blood vessels, each of which is thinner than a human hair. Usually, neither the number nor the size of these vessels increase, since the division of the endothelial cells covering the vessels is slow, actually up to several years. The exceptions are for example during wound healing and menstruation, when the vessels grow rapidly. However, that is during a limited period of time and the cell division ceases thereafter.

15   The generation of new blood vessels from existing ones is called angiogenesis. Angiogenesis has been associated to cancer and the formation of tumors as well as to other conditions, such as diabetes retinopathy, rheumatoid arthritis and even some inflammatory conditions. Accordingly, a considerable research effort is made world-wide to find ways of preventing and inhibiting the angiogenic process. If this were possible, tumor growth could
20   be controlled and useful therapies could be developed regarding the above mentioned conditions.

There exists numerous pieces of evidence showing that tumors are depending on *de novo* formation of blood vessels for expansion beyond a mass of a few mm^3. The angiogenesis is triggered by factors secreted by the tumor cells. It has recently been
25   discovered that tumors through unleashed proteolytic activity generates peptide fragments, which show anti-angiogenic activities. One example is the molecule angiostatin, which is a fragment of plasminogen.

Plasminogen is a substance in blood plasma which, when activated, forms plasmin or fibrinolysin, an enzyme involved in the coagulation of blood. Plasminogen itself lacks any
30   detectable anti-angiogenic activity. It has been found (Judah Folkman et al, Harvard Medical School, Boston) that a part of this endogenous protein, more specifically the first four kringle domains, is capable of preventing the endothelial cells from dividing. This part

of plasminogen has been denoted angiostatin, and a great deal of research within this field is centred around this molecule. The prior art has shows that angiostatin inhibits endothelial cells specifically *in vitro* and blocks angiogenesis *in vivo*. Systemic treatments with subcutaneous injections of angiostatin induces *in vivo* dormancy in a wide range of tumors

5   in SCID mice (O'Reilly et al., Nature Med., vol. 2, p. 689, 1996). No detectable toxicity has been detected in these animals even after months of treatment. Angiostatin shows two levels of specificity: it induces apoptosis specifically in endothelial cells *in vitro* (Claesson-Welsh et al., Proc. Natl. Acad. Sci., USA, vol. 95, p. 5579, 1998) and only affects endothelial cells active in angiogenesis *in vivo*. It has not shown to negatively affect cells in

10 established vessels.

Angiostatin does indeed exhibit some advantageous properties, *inter alia* as it is an endogenous substance. However, the disadvantages associated with its possible use for medical purposes cannot be neglected. One is that the half life thereof is very short, it may be counted in hours, thereby requiring a frequent administration thereof. This far, the

15 efficiency thereof has proven to be rather low, which fact necessitates the use of large doses thereof. These two disadvantages are in themselves strong motives for directing further research towards the finding of alternative, smaller and/or more efficient molecules to be used as medicaments.

20   Summary of the invention

The present invention solves the problems associated with angiostatin as defined above by providing a human protein, which has been named "ABP-1", defined by its ability to bind a fragment of plasminogen, preferably the first four Kringle domains (K1-K4) thereof, the said fragment being characterized by anti-angiogenic biological activity.

25   ABP-1 comprises an amino acid sequence substantially similar to that shown in SEQ ID NO. 2. Variants and fragments of ABP-1 are encompassed in the present invention.

Also encompassed in the present invention are the homologs of ABP-1 in other species, especially in other mammals.

In a further aspect, the invention provides isolated nucleic acid molecules comprising

30   a sequence that codes for ABP-1 or for a polypeptide substantially similar to ABP-1, including its variants, fragments and homologs.

It is another object of the present invention to provide nucleic acid probes whose sequence is derived from SEQ ID NO.1; these probes may be used as research tools as well as in diagnostic methods, for example to detect and measure ABP-1 biosynthesis in tissues and cells.

5 Accordingly, it is an object of the present invention to provide a diagnostic method for detecting the presence and the amount of ABP-1 or its variants and fragments in tissues and cells.

The present invention also includes screening methods for identifying a compound capable of interacting with ABP-1 or its variants and fragments. The screening method can 10 be in any configuration well known to those skilled in the art.

It is a further object of the present invention to provide compounds identified with the said screening method and capable of modulating the biological activity of ABP-1 or its variants and fragments.

It is a further object of the present invention to provide a pharmaceutical composition 15 comprising as active ingredient the compound identified with the above screening method.

It is still a further object of the present invention to provide antibodies directed against epitopes present in ABP-1 or its variants and fragments as well as cell producing the antibody.

It is a further object of the present invention to provide a vector comprising the 20 nucleotide sequence of ABP-1 or its variants and fragments.

It is a further object of the present invention to provide a cell containing the above vector.

Definitions

25 In the present application, the following terms are used in the meanings defined below.

As used herein, the term "angiogenesis" relates to the generation of new blood vessels into a tissue or organ. As mentioned above, under normal physiological conditions, humans and animals undergo angiogenesis only in very specific restricted situations. For example, 30 angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum and placenta.

The term "endothelium" relates to the thin layer of flat epithelial cells, that lines serous cavities, lymph vessels and blood vessels. The term "endothelial inhibiting activity" relates to the capability of inhibiting the growth of endothelial capillary endothelial cells.

The term "an angiogenesis associated protein" relates to a protein capable of inter-
acting in the angiogenesis, such as a receptor binding an anti-angiogenic substance.

The term "substantially similar", when used in reference to the amino acid sequence of SEQ ID NO.2, SEQ ID NO.3 and SEQ ID NO.4, means an amino acid sequence having an high degree of sequence homology to SEQ ID NO.2, SEQ ID NO.3 and SEQ ID NO.4. A high degree of homology means at least approximately 80% amino acid homology, preferably at least approximately 90% amino acid homology, more preferably at least approximately 95% amino acid homology and most preferably at least approximately 98% amino acid homology.

The term "specifically hybridizing to" refers to the binding, duplexing or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when the sequence is present in a complex mixture (e.g. total cellular) of DNA or RNA. The term "stringent conditions" relates to conditions under which a probe will hybridize to its target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at Tm, 50% of the probes are occupied at equilibrium). Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for longer probes. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

Brief description of the drawings

Figure 1 shows the binding of ^{125}I-labelled angiostatin and plasminogen to bovine microcapillary endothelial cells *in vitro*. Units in abscissa represent the fold molar excess of cold angiostatin and plasminogen, respectively.

~~Figure 2 shows the strategy for isolating angiostatin binding proteins.~~

Figure 3 shows the growth of positive yeast clones under selective conditions.

Figure 4 compares the binding of recombinant "Big-3" to angiostatin and plasminogen, respectively.

Figure 5 is a map over "Big-3".

Figure 6 shows the open reading frame (Frame 2) of the gene encoding the angiostatin receptor according to the invention. The other possible frames do not produce any putative protein.

Figure 7 shows the expression patterns of fetal and adult mRNA as well as endothelial cells.

Figure 8 shows the relative quantitation of ABP-1 gene expression in different tissue. See experimental section for more details.

Figure 9 illustrates (A) the cellular localization of GFP-tagged ABP-1 receptor in transiently transfected HeLa cells and (B) the reorganization of GFP-labeled ABP-1 after incubation with angiostatin. Figure 9C shows the binding of angiostatin to ABP-1. See the experimental section for more details. Figure 9D is an immunostaining of ABP-1 in Human umbilical cord endothelial (HUVE) cells together with staining against F-actin with rhodamin-labelled phalloidin. ABP-1 is localized in focal adhesions and membrane ruffles (arrows).

Figure 10 illustrates the down regulation of ABP-1 associated kinase activity after addition of angiostatin.

Figure 11 is an autoradiography of an SDS-PAGE demonstrating that ABP-1 mediates angiostatin-induced focal adhesion kinase activity. See experimental section for more details.

Detailed description of the invention

The present invention relates to an isolated human angiogenesis-associated protein capable of binding a fragment of plasminogen, preferably an N-terminal fragment, such as

kringle domains 1 to 4 and/or kringle 5. Thus, the protein of the invention acts as a receptor of plasminogen fragments, in particular as a receptor of angiostatin and/or kringle 5 domain of plasminogen. The protein of the invention can be synthesized by biological or chemical methods (e.g. recombinant gene expression and peptide synthesis). Recombinant techniques

5   include gene cloning or amplification by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS) or the self-sustained sequence replication system (SSR). A wide variety of cloning and *in vitro* amplification methodologies are well-known to those skilled in the art. Examples of these techniques are *e.g.* found in Berger and Kimmel, *Guide to Molecular*

10   *Cloning Techniques*, Methods in Enzymology 152 Academic Press, Inc., San Diego, CA (Berger). The present invention includes proteins which comprise amino acid sequences substantially similar to those shown in SEQ ID NO.2, SEQ ID NO.3 and SEQ ID NO.4. A comparison of the protein sequence of SEQ ID NO. 2 with the sequences present in all available data bases showed a homology of 23.7% with a hypotetical protein of

15   *Caenorhabditis elegans* encoded by a putative open reading frame located in the central cluster of chromosome III of this organism (described in R. Wilson et al., Nature, vol.368, 3 March 1994, p. 32-38). No function has been attributed to this hypotetical protein.

According to the definition given above, it is to be understood that all polypeptides capable of binding a fragment of plasminogen and having an amino acid sequence which

20   has at least approximately 80% sequence homology, preferably approximately 90% sequence homology, more preferably approximately 95% sequence homology and most preferably approximately 98% sequence homology to SEQ ID Nos.2, 3 or 4, are contemplated as being included in the present invention. These variant forms may result, e.g., from alternative splicing or differential expression in different tissue of the same

25   source organism. The variant forms may be characterized by, e.g., amino acid insertion, deletion or substitution. A preferred variant form of ABP-1 is illustrated in SEQ ID NO. 3 (see below).

A particularly preferred embodiment of the present invention is a fragment of ABP-1 named Big-3 which will be described in greater details below and which includes the

30   angiostatin-binding domain of ABP-1 (SEQ ID NO. 4).

In a further embodiment, the present invention provides peptides which comprises at least about 5, preferably at least about 10 contiguous amino acid residues of SEQ ID NO 2

or any variant or fragment thereof. However, the most advantageous size of the peptide will be depending on the intended future use thereof and the present invention is not limited to the above stated number of amino acids residues.

Also included in the present invention are homologs of ABP-1 and Big-3 in other
5 species, in particular in other mammals. The term "homolog" refers to proteins exerting substantially the same biological function of the proteins of the invention, regardless of the homology existing between the corresponding amino acid sequences.

In another aspect, the present invention relates to isolated nucleic acid molecules comprising a sequence that codes for the proteins and peptides of the invention, including
10 the variants, fragments and homologs as defined above. In a preferred embodiment, the nucleic acid molecule has the sequence of SEQ ID NO. 1. This sequence presents a 5' (from nucleotide 1 to nucleotide 796) and a 3' (from nucleotide 2825 to nucleotide 6463) untranslated regions. In another preferred embodiment the nucleic acid molecule has the sequence from nucleotide 797 to nucleotide 2824 of SEQ ID NO. 1. In another preferred
15 embodiment the nucleic acid sequence encodes the ABP-1 fragment named Big-3 (illustrated in SEQ ID NO. 4) and it comprises the nucleotide sequence from position 2180 to position 2608 of SEQ ID NO. 1. All the nucleotide sequences encoding the polypeptides of the invention and differing from the nucleotide sequence of SEQ ID NO. 1 or fragment thereof by way of the degeneracy of the genetic code, are considered part of the invention.
20 Sequencing analysis has revealed the presence of a possible polymorfism in the codon 1199-1201 of SEQ ID NO. 1, wherein a codon for Asn, Ser or Asp may be present; a further region corresponding to nucleotide positions 1238 to 1246 of SEQ ID NO. 1 has been found to code for the tripeptide Glu-Leu-Ala or for the tripeptide Thr-Trp-Pro. These variations in the amino acid sequence of ABP-1 are illustrated in SEQ ID NO. 3 which constitutes
25 another preferred protein of the invention. Also included in the present invention are nucleotide molecules encoding a homolog of ABP-1 or fragment thereof.

The present invention further comprises a nucleic acid capable of specifically hybridizing, under stringent conditions, to any one of the nucleic acid molecules of the invention described above.

30 Where the nucleic acids according to the invention are to be used as probes, it is often desirable to label the sequences with detectable markers. Such markers may include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical,

electrical, optical or chemical means. The markers may be incorporated by any of a number of means well known to those of skill in the art. Methods for detecting such markers are also well known in the art and disclosed in the literature. The probes find a useful application in diagnostic application, for example to detect and measure ABP-1 biosynthesis in tissues and cells.

In another aspect, the present invention provide screening methods or assays, wherein molecules that exhibit the same properties as angiostatin and/or kringle 5 are screened for. In a typical screening method a compound capable of activating the angiostatin signal transduction pathway is identified through a high throughput cell-based screen. Such screen rely on a reporter gene driven by an angiostatin responsive element that is stably transfected into an angiostatin-responsive cell line. The responsive element is linked to a reporter gene, e.g. the gene for luciferase; upon binding of the compound to the protein of the invention, the intracellular pathway is activated thus causing an increase in the reporter gene activity that can be detected.

The compounds identified through the screening method hereby described are within the scope of the present invention. Such compounds are preferably low molecular weight molecules of peptidic or non-peptidic nature. Thanks to their small size they are more practical for use for medicinal purposes, compared to angiostatin. These molecules and the uses thereof are described in more detail below.

The proteins and peptides according to the invention may be synthesized using standard chemical peptide synthesis techniques. For solid phase synthesis, see e.g. Barany and Merrifield, *Solid-Phase Peptide Synthesis*, pp 3-284 in *The Peptides*: Analysis, Synthesis Biology, Vol. 2: *Special Methods in Peptide Synthesis*, Part A.

Preferably, the proteins, peptides and polypeptides according to the invention are synthesized using recombinant DNA methodology, which generally involves creating a DNA sequence that encodes the protein or peptide, placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein or peptide in a host, isolating the expressed protein or peptide and, if required, renaturing the product. Once expressed, the recombinant peptides or proteins can be purified according to standard procedures in the art. Thus, another aspect of the present invention is a vector, such as a virus or a plasmid, comprising a nucleic acid according to the invention. Further, the invention also encompasses a recombinant cell transformed or transfected with the said

vector expressing the present protein or peptide as well as a recombinant cell expressing the present antibody, which will be defined in more detail below. Vectors encoding the peptides and proteins according to the invention are useful in expressing those molecules to provide immunogens for antibody production. The vectors according to the invention are also useful

5   for transforming cells *in vitro* or *in vivo* to express the present peptides and proteins. Cells expressing any one of the present nucleic acids, such as the gene defined by SEQ ID NO 1, may be used in a wide variety of contexts and are also within the scope of the present invention. Such cells may be eucaryotic or procaryotic.

The proteins and peptides according to the invention can be used as antigens for

10   raising antibodies against the same. Consequently, the invention also encompasses an antibody which specifically binds a peptide according to the invention. Such antibodies are useful for immunoassays, e.g. for the isolation of peptides or polypeptides. The peptides according to the invention may also be used in assays, such as amplification specific assays, immunological assays *etc*.

15   The antibodies according to the invention may be monoclonal or polyclonal and include individual, allelic, strain or species variants, or fragments thereof, both in their naturally occurring (full-length) forms and recombinant forms. Additionally, the antibodies are raised to the present peptides or polypeptides in either their native configuration or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods

20   of making antibodies are known to persons skilled in the art. For techniques for preparing monoclonal antibodies, see *e.g.* Stiites et al (eds.), *Basic and Clinical Immunology* (4th ed), Lange Medical Publications, Los Altos, CA, and references cited therein. For techniques that involve selection of libraries of recombinant antibodies in phage or similar vectors, see *e.g.* Huse *et al.* (1989) *Science* 246:1275-1281.

25   The molecules according to the invention may be used in pharmaceutical preparations, especially for the treatment and/or prevention of angiogenesis related disorders. Accordingly, the invention relates to a peptide, polypeptide, protein or antibody according to the invention for use as a medicament as well as to the use of said molecules in the manufacture of a medicament directed towards an angiogenesis related disease or disorder.

30   The invention also relates to a pharmacological preparation comprising a molecule according to the invention together with a pharmaceutically acceptable carrier. The molecules used as medicaments according to the invention may be anyone of the above

described peptides, polypeptides, proteins or antibodies as well as any novel substance identified in a screening method using the same and described above.

Accordingly, the most preferred use of the present molecules is in assays, wherein novel substances are screened for. Compounds may be identified which exhibit similar anti-angiogenic effects as angiostatin and/or kringle 5, but which are smaller, more efficient and preferably exhibits a longer half time in a human or animal body than angiostatin. The shorter half time may be due to a lower tendency to be degraded by proteases. When an organic compound is designed, a molecule according to the invention is used as a "lead" compound. The design of mimetics to known pharmaceutically active compounds is a well known approach in the development of pharmaceuticals based on such "lead" compounds. Mimetic design, synthesis and testing are generally used to avoid randomly screening a large number of molecules for a target property.

Thus, such novel molecules are preferably used as medicaments that may be administered in much lower doses than angiostatin, and which may be administered less frequently, such as e.g. once every fourteen days, which is to be compared to the half time of angiostatin which is about ten times shorter. In a particular embodiment, the novel molecules identified by the screening methods according to the invention are low molecular weight organic molecules, in which case a pharmaceutical preparation may be prepared thereof for oral intake, such as in tablets.

The pharmaceutical preparations according to the invention may however be prepared for any route of administration, e.g oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular or intraperitoneal. The nature of the carrier or other ingredients will depend on the specific route of administration. (Examples of techniques and protocols that are useful in this context are inter alia found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A (ed.), 1980.)

The dosage of these low molecular weight compounds will depend on the disease state or condition to be treted and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound For treating human or animals, between approximately 0.5 mg/Kg of body weight to 500 mg/Kg of body weight of the compound can be administered.

The present compounds and methods are advantageously used in relation to all kind of angiogenesis related disorders and/or diseases, such as tumor conditions, diabetes,

rheumatoid arthritis and even some inflammatory diseases, such as psoriasis, chronic inflammations of the intestines, asthma etc. It is also suggested that they may be used in order to treat and cure, or prevent, obesity.

In a particular embodiment, the present molecules are used in gene therapy. For a review of gene therapy procedures, see e.g. Anderson, *Science* (1992) 256:808-813.

A further advantageous use of the present invention is to develop methods of regulating the signalling of the present angiostatin and/or kringle 5 receptor in the body and therefore, the invention also relates to methods of treating a human or animal patient suffering from an angiogenesis related disease or disorder as well as to methods preventing such conditions.

EXPERIMENTAL

Below, the invention will be disclosed in more detail with reference to the drawings.

All references in the present disclosure and above are hereby incorporated in the present application.

Evidence that angiostatin binds to endothelial cells

By competitive binding assays between angiostatin and the angiogenic factors, VEGF and bFGF, the present inventors have shown that angiostatin does not affect the ligand-receptor interaction of these molecules. The same level of binding could be detected in the presence or absence of unlabeled angiostatin. Thus, it can be ruled out that angiostatin acts by blocking angiogenic factors interaction with endothelial cells.

The direct binding of angiostatin and its precursor plasminogen has been studied by binding assays using iodinated proteins and analyzing their interaction with bovine microcapillary endothelial cells, see Figure 1.

In particular, human angiostatin (kringle domains 1-4) or plasminogen was labeled with iodine 125 by the Iodogen method according to the protocol of the manufacturer (Pierce Inc.). The labelled protein was then purified on a G50 sepharose column (Pharmacia Inc.) The specific activity was estimated at 90 000 cpm/ng protein. For binding assays, bovine capillary endothelial cells were grown to confluency in 12 well plates. The cells were washed with PBS containing 1mg/ml bovine serum albumine (BSA). The cells were then incubated with 10ng/ml radio-labeled angiostatin or plasminogen. The binding was

competed with increasing concentrations of unlabeled ligand. Cells were incubated on ice for 2 hours. Cells were then washed five times with PBS+1mg/ml BSA. The cells were lysed with 1% Triton X100 in PBS and radioactivity was measured in a gamma counter. Dissociation constant and receptor numbers were estimated using the RBINDING program

5   (van Zoelen EJ. Anal. Biochem. 1992 Feb 1;200 (2):393-9).

Identification of angiostatin binding molecules using the yeast two hybrid system

Angiostatin retains activity even after reduction, suggesting that three dimensional folding is not vital for binding and activity. This favours screening procedures wherein a

10   large number of clones may be screened although protein refolding may be less accurate.

The yeast two hybrid system was employed to screen for molecules that bind kringle domains 1-4 of plasminogen. This is further disclosed below with reference to figure 2.

Approximately $2x10^6$ clones from a two hybrid cDNA library from human term placenta (Stratagene) were screened. Positives clones were identified in the following way:

15   (1) Screening under selective conditions (His-, Leu- and Trp-) generated 37 positive clones in yeast strain CG1945.

(2) Seven out of 37 clones displayed high β-galactosidase activity after incubation with ONPG (SIGMA) at 30^0C for 2h.

(3) The DNA from the seven colonies that contained high β-gal activity was

20   purified and retransfected into yeast strain Y190. Three out of the seven colonies retained activity in the new yeast strain. Sequencing analysis of these clones revealed that they were derived from the same gene.

(4) Growth in the presence of 50mM 3-aminotriazol (Fig. 3) as well as β-gal activity (see table below) was assessed in Big 3 clones and compared to positive and

25   negative controls.

Binding Domain	Activation Domain	β-Gal Activity
Angiostatin + Binding Domain	Big3 - Activation Domain	15 u
Angiostatin + Binding Domain	Activation Domain alone	<0.04 u
Binding Domain alone	Big3 - Activation Domain	0.07 u
p53 + Binding Domain	Big3 - Activation Domain	0.05 u
p53 + Binding Domain	SV40LT - Activation Domain	90 u

Sequence of Big 3 (angiostatin-binding domain)

After isolation of the big 3 clone from a placenta yeast two-hybrid library (Stratagene, Inc.), we directly sequenced the cDNA insert from the pGAD activation domain vector with GAL4 AD sequencing primers. The cDNA insert was recloned into pUC18 vector using EcoR1 sites from adaptors and sequencing was repeated using universal and reverse primers. Sequences were analyzed in an ALF automated sequencer (Pharmacia). The sequence of Big 3 is shown in SEQ ID NO. 4.

Expression and purification of Big3 and Big3-GST fusion protein

Big3 sequence has been expressed in *E.coli* as fusion protein with the glutathione S - transferase (GST) domain from *Schistosoma japonicum*.

●*Vector construction*:

Big3 fragment (429 bp) was obtained by PCR amplification using pUC18-Big3 plasmid as template. Primer sequences were:

Bam*HI*-NH₂   5' TAC GGA TCC GAA TCG AAC AAA ACT GCA GCT G 3' (SEQ ID NO. 5)

Xho*I*- COOH   5' ATA CTC GAG TCA TGG AGC TGG AGT TGG AGC CA 3' (SEQ ID NO. 6)

The cycling protocol used was:

94°C 3', 60°C 1', 72°C 1'	1cycle
94°C 30'', 60°C 1', 72°C 2'	30 cycles
94°C 30'', 60°C 1', 72°C 5'	1cycle

Taq polymerase: native Pfu DNA polymerase (Stratagene)

PCR fragment was digested with Bam*HI* and Xho*I*, purified and ligated to PGEX-6P-2 plasmid (Pharmacia Biotech) digested with the same restriction enzymes. Ligation mixture was used to transform XL1-Blue cells (Stratagene). Recombinant plasmids were verified by restriction analysis and automated sequencing.

●*Expression and purification*:

DH5α cells (Clontech) were transformed with one verified clone, named pGEX-Big3, and induced for 6 hours, room temperature with 0.1 mM IPTG.

The fermentation broth was centrifuged at 4000xrpm 15 min and the cell pellet was resuspended in 10% w/v of lysis buffer (50 mM TRIS.HCl pH 8.0, 100 mM NaCl, 20 mM

DTT , 1 mM EDTA, protease inhibitor mix), and lysed by sonication. The total lysate was centrifuged at 15000 xg for 20 min at 4°C. The clarified supernatant was applied to a Glutathione-Sepharose column (1ml for 20 ml of lysate) preequilibrated with lysis buffer. The resin was washed with 10 column volumes (CV) of lysis buffer and the fusion protein

5   was eluted with 3 CV of elution buffer (100 mM TRIS.HCl pH 8.0 20 mM reduced glutathione).

GST-Big3 protein can be cleaved by incubation of the glutathione-Sepharose bound fusion protein with 20 ul/ml resin of PreScission protease in PS buffer containing 50 mM TRIS.HCl pH 7,0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF.

10   The eluted proteins behave like a single peak in rp-HPLC (10-90% gradient of acetonitrile in water plus 0,1% TFA), show the expected molecular size by mass analysis (electrospray) and the correct NH2 sequence. In SDS-PAGE a contaminant appears at the apparent MW of 80 kD; it was demonstrated to be DnaK, a bacterial chaperone, which can be eliminated running an ion exchange chromatography on a HiTrapQ column.

15   The yields obtained from 1 litre of fermentation are about 10 mg of GST-Big3 fusion protein and about 3 mg of the cleaved Big3.

In vitro binding of recombinant GST-tagged Big 3 to angiostatin

The protocol used for binding is described here below.

20   One colony of pGEX-Big3 transformant was inoculated into 10 ml LB medium plus ampicillin and grown overnight at 37°C.

The overnight culture was diluted 1:10 into 100 ml fresh medium and after 1hour of incubation at 37°C, IPTG was added to a final concentration of 0.5mM and incubation continued for 3 to 7 hours.

25   The culture was then centrifuged at 5000g for 10 minutes, the pellet was resuspended in 3-5 ml of ice-cold PBS and cells were lysed by sonication. Triton X100 was added to a final concentration of 0.5 - 1 % and 1.5 ml aliquots of the suspension were centrifuged at 14000 rpm for10 minutes.

0.1-0.3ml of 50% slurry of glutathione-agarose.beads were added to the supernatant

30   and mixed for 3-5 hour at 4°C. The beads were then collected by centrifugation and washed 5 times with ice-cold PBS and 2 times with binding solution (B.S.) consisting of: 50mM Tris pH7.5, 150mM NaCl, 0.5mM EDTA, 1mM DTT,1mM PMSF).

Binding assay: 0.7ml B.S., 0.1% calf serum, 500ng angiostatin were mixed with 30-50μl 50% slurry of Big3-immobilized glutathione-agarose beads for 1 - 3 hour at 4°C.

The resin was washed 5-7 times with ice cold B.S., then used for Western blot analysis using anti-human plasminogen antibodies (Dako Inc.).

5   Figure 4 shows how purified recombinant Big 3 binds angiostatin in vitro. Interestingly, binding of plasminogen could also be detected, but only after reduction of disulphide bonds by treating it with DTT.

Cloning of full-length sequence

10   Figure 5 illustrates the scheme for cloning the full-length sequence of ABP-1. The whole gene was cloned by screening a placenta cDNA phage library with a Big3 probe (with repetitive sequences removed) together with 5' RACE PCR (Gibco) using mRNA from human umbilical cord endothelial cells. The full sequence of the cDNA clone is

15   disclosed in SEQ ID NO. 1, whereas the encoded protein is shown in SEQ ID NO. 2. Details of the experimental protocol are as follows.

10 million clones of a placenta lambda phage library (Stratagene, Inc.) were screened using a Hin*fI* fragment of Big 3 as probe. DNA isolation and Southern blot procedures were performed according to established protocols (Sambrook et al 1989). We isolated 5 clones

20   (7-1, 7-2, 8-2, 9-3, 9-5) with sequences that overlap with Big3. The 5'-sequence of the 7-2 clone was used for designing Gene-Specific Primers (GSP) for 5'RACE PCR (Gibco Life technologies, Inc.). mRNA isolated from Human umbilical cord endothelial cells was used to identify 5' sequences.

Primers used for the first RACE PCR:

25   Primers: GSP1- (5' to 3') GCTGACAGTTGCCCTGACGCTGCT (SEQ ID NO. 10)
            GSP2- (5' to 3') CGGAGACGGTGCTCTAGCTGCTCA (SEQ ID NO. 11)
            GSP3- (5' to 3') TCCTTCCAACTCTTGCCTCAAGTTCCG (SEQ ID NO. 12)

RACE procedures have been used for amplification and cloning unknown sequences

30   between the GSP2 and GSP3 and the 5'-end of the mRNA ABP-1. This sequence was then used to design new primers for the next set of RACE RCR:

Primers: GSP1-(5' to 3') GGTGGCAGCGGACAGGCAGGATAC (SEQ ID NO. 13)

GSP2   GAGGCGGAGAGAACTAAGAGAAGA (SEQ ID NO. 14)

GSP3   GAGCGGAGATGGAGGAGTAATTCA (SEQ ID NO. 15)

5   Clones A2-2, A2-1 and A1-C with overlapping sequences were isolated. The A2-1 sequence was used as probe for the second screening of the placenta phage library . From this screening we isolated another 2 clones (7-10 and 6-5). As shown in Figure 6, of the six possible frame the only one producing a complete open reading frame is frame 2, yielding a putative protein of 675 amino acid residues.

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mRNA Expression pattern

A commercially (Clontech, Inc.) obtained multiple human adult and fetal tissue (#7760-1 and #7756-1) mRNA blots (2mg mRNA/well) was probed for ABP-1. The blots were hybridized with the ExpressHyb hybridization solution (Clontech, Inc.) according to the

15   protocol of the manufacturer. The blots were probed with the 7-2 5'PstI fragment. Figure 7 shows the results of the experiment; sizes are 9.5 and 7.5 kb.

ABP-1 was also detected in human umbilical cord, bovine aortic and bovine microcapillary endothelial cells. Little or no expression was detected in the immortalized endothelial cell-line EaHy926 and in human fetal fibroblasts. Neither of the cell lines

20   respond to angiostatin or exhibit any binding of FITC-labeled angiostatin.

Real time RT-PCR

The 5' nuclease assay (TaqMan assay) uses a nonextendable oligonucleotide hybridization probe (TaqMan Probe). The TaqMan probe consists of an oligonucleotide

25   with a 5'-reporter dye and a 3'-quencher dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by a Foster-type energy transfer. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. The Taq Polymerase cleaves the TaqMan probe

30   with its 5'-3' nuclease activity. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter.

Real time RT-PCR analysis

RNA Preparation

5   Isolation and purification of total RNA from tissues and cells listed below was performed using the Ultraspec RNA isolation system (Biotex).

HDMEC/2	h.dermal microvascular EC	poly A+
A2780	h.ovarian carcinoma	TOTAL
A375	h. melanoma	TOTAL
HDF	H.dermal fibroblasts	TOTAL
HELA	h.cervix carcinoma	TOTAL
DU145	h.prostate carcinoma	TOTAL
HUVEC	h.umbelical vein EC	TOTAL
ECV304	immortalized EC	TOTAL
CEM	h.acutelymphobl.leuk.	TOTAL
K562	h.erythroleukemia	TOTAL
JURKAT	h.acuteT-cellleukemia	TOTAL
THP-1	h.monocyte(acute leu)	TOTAL
EaHy926	h.dermal cells	TOTAL
S35/K9	human colon cancer	TOTAL
Brain	human normal tissue	TOTAL
Colon	human normal tissue	TOTAL
Prostate	human normal tissue	TOTAL
Skel.Muscle	human normal tissue	TOTAL
Uterus	human normal tissue	TOTAL
Placenta	human normal tissue	TOTAL

10   ### cDNA Synthesis

The RT reaction was performed using TaqMan Reverse Transcription Reagent (PE Applied Biosystems) with Random hexamers, as RT primers. The reaction volume for Reverse Transcription step was 100 μl and the total RNA amount was 1 μg for sample.

The RT was performed using the following cycling parameter:

5

      10' at 25°C

      45' at 48°C

       5' at 95°C

10   <u>PCR reaction</u>

After a primer concentration optimization, the PCR reaction on 10 ng of the cDNA was performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems) and the following oligonucleotides

- ABP-1 Forward Primer: 5' GTTTGACCTGCAATCCAGACAA 3' (SEQ ID

15   NO. 7) Final concentration 300nM

- ABP-1 Reverse Primer: 5' CCCAGGATCTGAATGGGAGTT 3' (SEQ ID NO.

8) Final concentration 900nm

- ABP-1 TaqMan Probe:

5' (FAM dye)-CAGATGGGCCTGTGTTCCACTCCAA-(TAMRA dye) 3' (SEQ ID NO.

20   9) Final probe concentration 200nM

The cycling protocols was:

2' at 50°C; 10' at 95°C 1 cycle

15" at 95°C; 1' at 60°C 40 cycles

25

<u>Relative Quantitation of gene expression</u>

The Comparative method use an arithmetic formula to achieve the result for relative quantitation without need for Standard curve.

The result , depicted in Figure 8, indicate how many fold the sample X expresses the

30   target relative to the Calibrator which is the sample that shows the lowest level of expression of the target. In this experiment EaHY926 sample was chosen as calibrator.

Antibodies against Big-3

For immunisation of New Zealand White rabbits, 100 micrograms of Big3-GST fusion protein were dissolved in 1 ml phosphate buffered saline (PBS) homogenised with 1 ml of Freund's Complete Adjuvant (Gibco). The resulting emulsion was injected
5 subcutaneously on day 0 and this treatment was repeated on days 15 and 28. Blood was removed from rabbits on day 35, allowed to coagulate overnight at 4°C, and the resulting serum stored at -20°C.

For purification of specific antibodies, resin-immobilised ligand was produced as follows: Big3 polypeptide was diluted in 0.1M sodium bicarbonate, 0.5 M NaCl, pH8.3, at
10 a concentration of 5 mg/ml in a total volume of 2 ml. This was reacted for two hours at room temperature with 2 ml of cyanogen bromide-activated Sepaharose CL-4B resin (Sigma Chemical Co. St Louis, MO). After reaction, the resin was washed three times in 10 volumes of 100 mM Tris, 500 mM NaCl, pH 7.5. Immune serum was incubated with the affinity resin for two hours at 4°C, after which resin was washed in a 5 ml glass
15 chromatography column (Bio-Rad, Richmond CA)for with 25 volumes of 100 mM Tris, 500 mM NaCl, pH 7.5. Specific antibodies were eluted in 1 ml aliquots with 100 mM Glycine/HCl, pH 2.8. Elution of antibody was followed by monitoring the optical density at 280 nM (OD280), and fractions with an OD280 of greater than 1 were pooled and dialysed (Slide-A-lyzer cassettes, Pierce) for 36 hours at 4°C against 1 liter PBS, with three changes
20 of buffer.

Binding and signalling of angiostatin via the ABP-1 protein

Figures 9A and 9B show data of binding of FITC-labeled angiostatin to ABP-1 transfected HeLa and EaHy926 cells transfected with the vector control. In particular figure
25 9A shows the cellular localization of ABP-1 fused to green fluorescent protein (GFP) in transiently transfected cells. The protein can be detected in the endoplasmic reticulum and the cell membrane. DABP-1 contains a 500bp deletion in the 5'end of the gene, which disrupts the previously described localization of ABP-1.

Figure 9B shows HeLa cells transfected with ABP-1 GFP. Incubation with 2.5 mg of
30 angiostatin for 60 minutes at 0°C and subsequent incubation for 15 minutes at 37°C induces internalization of ABP-1-GFP.

Figure 9C shows the binding of angiostatin to ABP-1. We have shown that fluorescein isothiocyanate (FITC)-labelled angiostatin binds specifically to endothelial cells (data not shown). We have transfected HeLa cells with either the ABP-1 expression construct or the vector control (p RC/CMV, In Vitrogen, Inc.). We incubated live HeLa cells FITC-labelled angiostatin (10ug/ml) in DMEM+10% fetal calf serum at 0°C for 60 minutes. The cells were then incubated at 37°C at 15 minutes to aggregate bound angiostatin. Binding was analyzed with a fluorescent microscope. Binding of angiostatin was detected in ABP-1 transfected cells but not in the vector control.

Figure 9D shows immunostaining of ABP-1 in Human umbilical cord endothelial (HUVE) cells together with staining against F-actin with rhodamin-labelled phalloidin.

• *Immunostaining protocol:*

HUVE cells were fixed in 4 % formaldehyde, washed in PBS and preblocked in 5% horse serum. The blocking solution was removed and replaced by rabbit polyclonal antibodies against the angiostatin-binding domain Big3 diluted in 5% horse serum. Positive staining was visualized with a fluorescence-labeled secondary antibody (Dako, Inc.). Rhodamin-conjugated phalloidin (Molecular Probes, Inc.) was stained simultaneously with the secondary antibody (after permeabilization with 1%triton X100 for 1 minute). Rabbit polyclonal antibodies against green fluorescent protein was used as a negative control. In addition no positive staining could be detected in human fibroblasts.

As can be seen in Fig. 9D, ABP-1 is localized in focal adhesions and membrane ruffles (arrows).

In vitro kinase data

Huve cells were plated subconfluent in 5cm Petri dishes. Angiostatin was added at 2.5 mg/ml at different time points the following day. All plates including controls were harvested simultanously. The cells were rinsed in ice cold PBS and incubated in 1ml lysis buffer. The cells were transferred to an eppendorf tube and centrifuged at 14 000rpm for 5 minutes at 4°C. The supernatant was transferred to another eppendorf tube and 2μg of Big 3 rabbit polyclonal antibodies was added. The samples were incubated for 60 min. at 4°C and subsequently 50μl of a protein A sepharose (Pharmacia, Inc.) slurry was added and incubated for another 60 min. in a rotating incubator. The immunoprecipitates were collected by brief centrifugation and washed two times in lysis buffer, once in washing

buffer and once in kinase buffer. Residual buffer was removed with a syringe. 25 µl of

kinase buffer was added to each tube together with 1µCi gammaATP (Amersham, Inc.).

The samples were incubated at R.T. for 20 minutes. The reaction was stopped by adding

2xSDS PAGE sample buffer. The samples were boiled under denaturing conditions and

5   subsequently analyzed by SDS PAGE.   *K 1-4*

The data illustrated in Figure 10 show that addition of angiostatin downregulates ABP-1

associated kinase activity within 30 minutes. This is direct proof that angiostatin affects

signalling pathways that are mediated by ABP-1.

10   *ABP-1 mediates Angiostatin-induced focal adhesion kinase activity.*

The data illustrated by Figure 11 show that addition of 2.5 µg/ml of angiostatin

upregulates FAK activity within 1 hour after addition.

EaHy926 cells were plated subconfluent in 5cm Petri dishes. Angiostatin was added

at 2.5 µg/ml at different time points the following day. All plates including controls were

15   harvested simultaneously. The cells were rinsed in ice cold PBS and incubated in 1ml lysis

buffer. The cells were transferred to an eppendorf tube and centrifuged at 14 000rpm for 5

minutes at 4°C. The supernatant was transferred to another eppendorf tube and 1ug of FAK

monoclonal antibody (Transduction Lab. Inc.) was added. The samples were incubated for

60 min. at 4°C and subsequently rabbit anti-mouse IgG + 50µl of a protein A sepharose

20   (Pharmacia, Inc.) slurry was added and incubated for another 60 min. in a rotating

incubator. The immunoprecipitates were collected by brief centrifugation and washed two

times in lysis buffer, once in washing buffer and once in kinase buffer. Residual buffer was

removed with a syringe. 25 µl of kinase buffer was added to each tube together with 1µCi

γATP (Amersham, Inc.). The samples were incubated at R.T. for 20 minutes. The reaction

25   was stopped by adding 2xSDS PAGE sample buffer. The samples were boiled under

denaturing conditions and subsequently analyzed by SDS PAGE.

As can be seen in Fig. 11, addition of angiostatin upregulates FAK activity within 1

hour. It should be noted that EaHy926 cells are not expressing ABP1 as demonstrated by

reverse transcriptase PCR analysis.

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2

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cctaatgatt gtgaccccat tctgtaatca actgagctag ttccaataaa gttaagcagg 6454

tttaaatcc   6463

<210> 2
<211> 675
<212> PRT
<213> Homo sapiens

<400> 2
Met Pro Arg Ala Gln Pro Ser Ser Ala Ser Tyr Gln Pro Val Pro Ala
 1   5   10   15

Asp Pro Phe Ala Ile Val Ser Arg Ala Gln Gln Met Val Glu Ile Leu
            20   25   30

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Ser Asp Glu Asn Arg Asn Leu Arg Gln Glu Leu Glu Gly Cys Tyr Glu
        35                  40                  45

Lys Val Ala Arg Leu Gln Lys Val Glu Thr Glu Ile Gln Arg Val Ser
        50                  55                  60

Glu Ala Tyr Glu Asn Leu Val Lys Ser Ser Ser Lys Arg Glu Ala Leu
65                  70                  75                  80

Glu Lys Ala Met Arg Asn Lys Leu Glu Gly Glu Ile Arg Arg Met His
                85                  90                  95

Asp Phe Asn Arg Asp Leu Arg Glu Arg Leu Glu Thr Ala Asn Lys Gln
                100                 105                 110

Leu Ala Glu Lys Glu Tyr Glu Gly Ser Glu Asp Thr Arg Lys Thr Ile
        115                 120                 125

Ser Gln Leu Phe Ala Lys Asn Lys Glu Ser Gln Arg Glu Lys Glu Lys
        130                 135                 140

Leu Glu Ala Glu Leu Ala Thr Ala Arg Ser Thr Asn Glu Asp Gln Arg
145                 150                 155                 160

Arg His Ile Glu Ile Arg Asp Gln Ala Leu Ser Asn Ala Gln Ala Lys
                165                 170                 175

Val Val Lys Leu Glu Glu Glu Leu Lys Lys Lys Gln Val Tyr Val Asp
                180                 185                 190

Lys Val Glu Lys Met Gln Gln Ala Leu Val Gln Leu Gln Ala Ala Cys
        195                 200                 205

Glu Lys Arg Glu Gln Leu Glu His Arg Leu Arg Thr Arg Leu Glu Arg
        210                 215                 220

Glu Leu Glu Ser Leu Arg Ile Gln Gln Arg Gln Gly Asn Cys Gln Pro
225                 230                 235                 240

Thr Asn Val Ser Glu Tyr Asn Ala Ala Ala Leu Met Glu Leu Leu Arg
                245                 250                 255

Glu Lys Glu Glu Arg Ile Leu Ala Leu Glu Ala Asp Met Thr Lys Trp
        260                 265                 270

Glu Gln Lys Tyr Leu Glu Glu Asn Val Met Arg His Phe Ala Leu Asp
        275                 280                 285

Ala Ala Ala Thr Val Ala Ala Gln Arg Asp Thr Thr Val Ile Ser His
        290                 295                 300
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Ser Pro Asn Thr Ser Tyr Asp Thr Ala Leu Glu Ala Arg Ile Gln Lys
305   310   315   320

Glu Glu Glu Glu Ile Leu Met Ala Asn Lys Arg Cys Leu Asp Met Glu
              325   330   335

Gly Arg Ile Lys Thr Leu His Ala Gln Ile Ile Glu Lys Asp Ala Met
              340   345   350

Ile Lys Val Leu Gln Gln Arg Ser Arg Lys Glu Pro Ser Lys Thr Glu
              355   360   365

Gln Leu Ser Cys Met Arg Pro Ala Lys Ser Leu Met Ser Ile Ser Asn
370   375   380

Ala Gly Ser Gly Leu Leu Ser His Ser Ser Thr Leu Thr Gly Ser Pro
385   390   395   400

Ile Met Glu Glu Lys Arg Asp Asp Lys Ser Trp Lys Gly Ser Leu Gly
              405   410   415

Ile Leu Leu Gly Gly Asp Tyr Arg Ala Glu Tyr Val Pro Ser Thr Pro
              420   425   430

Ser Pro Val Pro Pro Ser Thr Pro Leu Leu Ser Ala His Ser Lys Thr
              435   440   445

Gly Ser Arg Asp Cys Ser Thr Gln Thr Glu Arg Gly Thr Glu Ser Asn
              450   455   460

Lys Thr Ala Ala Val Ala Pro Ile Ser Val Pro Ala Pro Val Ala Ala
465   470   475   480

Ala Ala Thr Ala Ala Ala Ile Thr Ala Thr Ala Ala Thr Ile Thr Thr
              485   490   495

Thr Met Val Ala Ala Ala Pro Val Ala Val Ala Ala Ala Ala Ala Pro
              500   505   510

Ala Ala Ala Ala Ala Pro Ser Pro Ala Thr Ala Ala Ala Thr Ala Ala
              515   520   525

Ala Val Ser Pro Ala Ala Ala Gly Gln Ile Pro Ala Ala Ala Ser Val
              530   535   540

Ala Ser Ala Ala Ala Val Ala Pro Ser Ala Ala Ala Ala Ala Val
545   550   555   560

Gln Val Ala Pro Ala Ala Pro Ala Pro Val Pro Ala Pro Ala Leu Val
              565   570   575

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Pro Val Pro Ala Pro Ala Ala Ala Gln Ala Ser Ala Pro Ala Gln Thr
        580             585             590

Gln Ala Pro Thr Ser Ala Pro Ala Val Ala Pro Thr Pro Ala Pro Thr
        595             600             605

Pro Thr Pro Ala Val Ala Gln Ala Glu Val Pro Ala Ser Pro Ala Thr
    610             615             620

Gly Pro Gly Pro His Arg Leu Ser Ile Pro Ser Leu Thr Cys Asn Pro
625             630             635             640

Asp Lys Thr Asp Gly Pro Val Phe His Ser Asn Thr Leu Glu Arg Lys
            645             650             655

Thr Pro Ile Gln Ile Leu Gly Gln Glu Pro Asp Ala Glu Met Val Glu
            660             665             670

Tyr Leu Ile
        675


<210> 3
<211> 675
<212> PRT
<213> Homo sapiens

<220>
<221> VARIANT
<222> ()..)
<223> Residue 135 = Asn, Ser or Asp

<220>
<221> VARIANT
<222> ()..(150)
<223> Residues 148-150 = Glu-Leu-Ala or Thr-Thr-Pro

<400> 3
Met Pro Arg Ala Gln Pro Ser Ser Ala Ser Tyr Gln Pro Val Pro Ala
 1               5               10              15

Asp Pro Phe Ala Ile Val Ser Arg Ala Gln Gln Met Val Glu Ile Leu
            20              25              30

Ser Asp Glu Asn Arg Asn Leu Arg Gln Glu Leu Glu Gly Cys Tyr Glu
        35              40              45

Lys Val Ala Arg Leu Gln Lys Val Glu Thr Glu Ile Gln Arg Val Ser
    50              55              60

Glu Ala Tyr Glu Asn Leu Val Lys Ser Ser Ser Lys Arg Glu Ala Leu
65              70              75              80
```

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Glu Lys Ala Met Arg Asn Lys Leu Glu Gly Glu Ile Arg Arg Met His
           85   90   95

Asp Phe Asn Arg Asp Leu Arg Glu Arg Leu Glu Thr Ala Asn Lys Gln
           100   105   110

Leu Ala Glu Lys Glu Tyr Glu Gly Ser Glu Asp Thr Arg Lys Thr Ile
      115   120   125

Ser Gln Leu Phe Ala Lys Xaa Lys Glu Ser Gln Arg Glu Lys Glu Lys
      130   135   140

Leu Glu Ala Xaa Xaa Xaa Thr Ala Arg Ser Thr Asn Glu Asp Gln Arg
145   150   155   160

Arg His Ile Glu Ile Arg Asp Gln Ala Leu Ser Asn Ala Gln Ala Lys
          165   170   175

Val Val Lys Leu Glu Glu Glu Leu Lys Lys Lys Gln Val Tyr Val Asp
          180   185   190

Lys Val Glu Lys Met Gln Gln Ala Leu Val Gln Leu Gln Ala Ala Cys
          195   200   205

Glu Lys Arg Glu Gln Leu Glu His Arg Leu Arg Thr Arg Leu Glu Arg
      210   215   220

Glu Leu Glu Ser Leu Arg Ile Gln Gln Arg Gln Gly Asn Cys Gln Pro
225   230   235   240

Thr Asn Val Ser Glu Tyr Asn Ala Ala Ala Leu Met Glu Leu Leu Arg
          245   250   255

Glu Lys Glu Glu Arg Ile Leu Ala Leu Glu Ala Asp Met Thr Lys Trp
          260   265   270

Glu Gln Lys Tyr Leu Glu Glu Asn Val Met Arg His Phe Ala Leu Asp
      275   280   285

Ala Ala Ala Thr Val Ala Ala Gln Arg Asp Thr Thr Val Ile Ser His
      290   295   300

Ser Pro Asn Thr Ser Tyr Asp Thr Ala Leu Glu Ala Arg Ile Gln Lys
305   310   315   320

Glu Glu Glu Glu Ile Leu Met Ala Asn Lys Arg Cys Leu Asp Met Glu
          325   330   335

Gly Arg Ile Lys Thr Leu His Ala Gln Ile Ile Glu Lys Asp Ala Met
      340   345   350

Ile Lys Val Leu Gln Gln Arg Ser Arg Lys Glu Pro Ser Lys Thr Glu
        355   360   365

Gln Leu Ser Cys Met Arg Pro Ala Lys Ser Leu Met Ser Ile Ser Asn
    370   375   380

Ala Gly Ser Gly Leu Leu Ser His Ser Ser Thr Leu Thr Gly Ser Pro
385   390   395   400

Ile Met Glu Glu Lys Arg Asp Asp Lys Ser Trp Lys Gly Ser Leu Gly
            405   410   415

Ile Leu Leu Gly Gly Asp Tyr Arg Ala Glu Tyr Val Pro Ser Thr Pro
        420   425   430

Ser Pro Val Pro Pro Ser Thr Pro Leu Leu Ser Ala His Ser Lys Thr
        435   440   445

Gly Ser Arg Asp Cys Ser Thr Gln Thr Glu Arg Gly Thr Glu Ser Asn
    450   455   460

Lys Thr Ala Ala Val Ala Pro Ile Ser Val Pro Ala Pro Val Ala Ala
465   470   475   480

Ala Ala Thr Ala Ala Ala Ile Thr Ala Thr Ala Ala Thr Ile Thr Thr
            485   490   495

Thr Met Val Ala Ala Ala Pro Val Ala Val Ala Ala Ala Ala Ala Pro
        500   505   510

Ala Ala Ala Ala Ala Pro Ser Pro Ala Thr Ala Ala Ala Thr Ala Ala
    515   520   525

Ala Val Ser Pro Ala Ala Ala Gly Gln Ile Pro Ala Ala Ala Ser Val
    530   535   540

Ala Ser Ala Ala Ala Val Ala Pro Ser Ala Ala Ala Ala Ala Ala Val
545   550   555   560

Gln Val Ala Pro Ala Ala Pro Ala Pro Val Pro Ala Pro Ala Leu Val
            565   570   575

Pro Val Pro Ala Pro Ala Ala Ala Gln Ala Ser Ala Pro Ala Gln Thr
        580   585   590

Gln Ala Pro Thr Ser Ala Pro Ala Val Ala Pro Thr Pro Ala Pro Thr
        595   600   605

Pro Thr Pro Ala Val Ala Gln Ala Glu Val Pro Ala Ser Pro Ala Thr
    610   615   620

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Gly Pro Gly Pro His Arg Leu Ser Ile Pro Ser Leu Thr Cys Asn Pro
625   630   635   640

Asp Lys Thr Asp Gly Pro Val Phe His Ser Asn Thr Leu Glu Arg Lys
                645   650   655

Thr Pro Ile Gln Ile Leu Gly Gln Glu Pro Asp Ala Glu Met Val Glu
                660   665   670

Tyr Leu Ile
        675

<210> 4
<211> 143
<212> PRT
<213> Homo sapiens

<400> 4
Glu Ser Asn Lys Thr Ala Ala Val Ala Pro Ile Ser Val Pro Ala Pro
  1   5   10   15

Val Ala Ala Ala Ala Thr Ala Ala Ala Ile Thr Ala Thr Ala Ala Thr
            20   25   30

Ile Thr Thr Thr Met Val Ala Ala Ala Pro Val Ala Val Ala Ala Ala
        35   40   45

Ala Ala Pro Ala Ala Ala Ala Ala Pro Ser Pro Ala Thr Ala Ala Ala
    50   55   60

Thr Ala Ala Ala Val Ser Pro Ala Ala Ala Gly Gln Ile Pro Ala Ala
65   70   75   80

Ala Ser Val Ala Ser Ala Ala Ala Val Ala Pro Ser Ala Ala Ala Ala
            85   90   95

Ala Ala Val Gln Val Ala Pro Ala Ala Pro Ala Pro Val Pro Ala Pro
            100   105   110

Ala Leu Val Pro Val Pro Ala Pro Ala Ala Ala Gln Ala Ser Ala Pro
        115   120   125

Ala Gln Thr Gln Ala Pro Thr Ser Ala Pro Ala Val Ala Pro Thr
    130   135   140

<210> 5
<211> 31
<212> DNA
<213> Artificial Sequence

13

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for PCR reaction

<400> 5
tacggatccg aatcgaacaa aactgcagct g   31

<210> 6
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for PCR reaction

<400> 6
atactcgagt catggagctg gagttggagc ca   32

<210> 7
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for PCR reaction

<400> 7
gtttgacctg caatccagac aa   22

<210> 8
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for PCR reaction

<400> 8
cccaggatct gaatgggagt t   21

<210> 9
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for PCR reaction

<400> 9
cagatgggcc tgtgttccac tccaa   25

<210> 10
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 10
gctgacagtt gccctgacgc tgct   24

<210> 11
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 11
cggagacggt gctctagctg ctca   24

<210> 12
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 12
tccttccaac tcttgcctca agttccg   27

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

15

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 13   24
ggtggcagcg gacaggcagg atac

<210> 14
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 14   24
gaggcggaga gaactaagag aaga

<210> 15
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide
      primer for RACE PCR reaction

<400> 15   24
gagcggagat ggaggagtaa ttca